IDENTIFICATION OF A NOVEL BIOMARKER FOR ORAL CANCERS USING SALIVA

DISSERTATION SUBMITTED FOR
MASTER OF SURGERY (BRANCH I)
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THE TAMILNADU
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CERTIFICATE

This is to certify that the dissertation entitled **"IDENTIFICATION OF A NOVEL BIOMARKER FOR ORAL CANCERS USING SALIVA"** submitted by **Dr.V.SUNIL KUMAR** to the Faculty of surgery, The Tamilnadu Dr. M.G.R. Medical university, Chennai in partial fulfillment of the requirement for the award of **M.S. Degree in SURGERY** is a bonafide work carried out by her during the period of August 2007 – July 2009 under my direct supervision and guidance.

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DECLARATION

I, Dr.V.SUNILKUMAR solemnly declare that the dissertation titled **"IDENTIFICATION OF A NOVEL BIOMARKER FOR ORAL CANCERS USING SALIVA"** has been prepared by me.

This is submitted to The Tamil Nadu Dr. M.G.R. Medical University, Chennai, in partial fulfillment of the requirement for the award of Master of Surgery, **(Branch I) General Surgery** Degree Examination to be held in March 2010.

Place : Madurai

Date :

DR. SUNIL KUMAR

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CONTENTS

INTRODUCTION

There are billions of cells in the human body. From the fertilized egg to death in old age, a human being is the product of billions of cell divisions. Like all complex systems, growth control can go wrong, resulting in the loss of normal territorial restraint, producing a family of cells that can multiply indefinitely- termed cancerous growth. It is not just the local growth of tumour cells that makes them so lethal but it is their spread, directly through invasion and by metastases to other sites of the body.

The prevalence cancer is often strikingly dissimilar in different groups of population, varies greatly from one community to another, and differs in different communities in the same geographical location, depending on the practices and lifestyles of the people in that location. Among various cancers affecting the human body, oral cancer accounts nearly one third of all. Oral cancer is one of the ten leading cancers in the world and shows marked geographic differences in occurrence.

Several factors have been considered responsible for the development of oral cancer. The use of tobacco, ill-fitting dentures, poor oral hygiene, syphilis, inadequate diet, malnutrition and chronic irritation from rough or broken teeth have been shown to be more frequent in oral cancer patients. However, among these factors use of tobacco stands first. More people use tobacco today than at any other time in human history. Tobacco is amongst the most addictive product known and this tobacco dependence is a progressive, chronic and relapsing disorder. Tobacco is the biggest killer, much bigger in dimension than all other forms of pollution. Tobacco kills more than AIDS, legal and illegal drugs, road accidents, murder and suicides combined. The smokers have markedly increased risk of multiple cancers, heart diseases, and strokes and if they chew tobacco, the risk of cancer of the lip, tongue and mouth increases.

The American Cancer Society's most recent estimates for oral cavity and oropharyngeal cancers in the United States are for 2009 about 28,500 new cases (20,100 in men and 8,400 in women) of oral cavity and oropharyngeal cancer; an estimated 6,100 people (4,200 men and 1,900 women) will die of these cancers

When patients newly diagnosed with oral and oropharyngeal cancers are carefully examined, about 15% will have another cancer in a nearby area such as the larynx, the esophagus or the lung. Of those who are cured of their oral or oropharyngeal cancer, 10% to 40% will develop another cancer later. Often, the cancer will occur in one of these organs or a new cancer will develop in the throat or mouth. For this reason, patients with oral and oropharyngeal cancer need to have follow-up exams for the rest of their lives. They also need to avoid using tobacco and alcohol, which increase the risk for these second cancers.

It is estimated that there are approximately 2 to 2.5 million cases of cancer in India at any given point of time and that there are 7,00,000 new cancer patients diagnosed every year in India. Tobacco-related cancer (TRC) cases constitute 48.2% in men and 20.1% in women of the total cancers seen in India per year. The age-adjusted rate (AAR) of TRC ranges from 44 to 67 among males and from 23 to 27 among females in different registries in India. The lifetime cumulative risk (0-74 years) of cancer in Madras is one in eight with age-adjusted rate (AAR) of oral cavity of 9.4. In India, oral cancers account for four in 10 of all cancers.

In a developing country like India, the second most populous country in the world, health is major issue. It is especially true in the rural population. The illiteracy, ignorance, poverty, lack of awareness, lack of access to treatment facilities including preventive measures and unequal distribution of health care facilities makes it a much more complicated and complex pubic health issue. Oral cancer presents a major health problem in India (15-70% of all cancers diagnosed are found in the oral cavity).

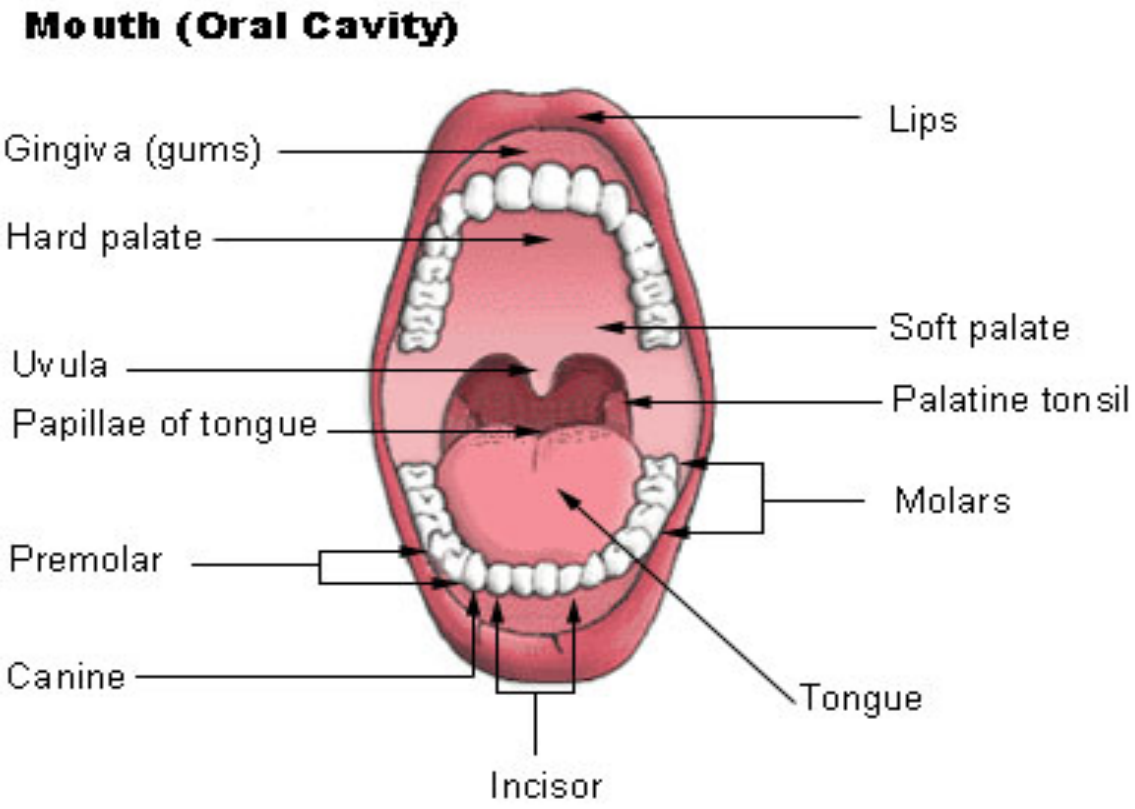
A *beedi* is an Indian form of cheap cigarette, a smoke for the common man in the country. It is made by rolling between the fingers a rectangular dried piece of Temburni (Diospyrosmelanoxylon), also called Tendu leaf with 0.30-0.36 gram of tobacco and securing the roll with a thread. The length of a beedi varies from 4 cm to 7.5 cm. *Chutta* is a more coarsely prepared cheroot and is often smoked with the burning end inside the mouth.

ANATOMY

ORAL CAVITY AND OROPHARYNX

ANATOMY OF ORAL CAVITY:

The oral cavity extends from the skin and vermillion junction of the lips to the junction of the hard and soft palate above and to the linea terminalis or line of circumvallate papillae on the tongue below.



Lips:

It is well defined into upper and lower lips that are joined laterally at the commissures. In the midline of the upper lip is the philtrum. They are supplied by the facial artery or branches of the external carotid system. Local spread of the tumour can occur along the adventitia of the facial vessels.

Lower lip is supplied by branches of the mandibular nerve and the upper lip by maxillary nerve.

Buccal Mucosa:

This area includes all the membranous lining of the inner surface of the cheeks and lips. It is supplied by the facial artery and maxillary artery. The nerve supply is by the mandibular nerve via the buccal nerve.

Upper Alveolar Ridge:

It is the alveolar process of the maxilla and its covering mucosa. The arterial supply is by the maxillary artery and the nerve supply is by branches of the maxillary nerve.

Lower Alveolar Ridge:

This includes alveolar process of the mandible and the covering mucosa. It is supplied by the lingual artery and maxillary artery. The nerve supply is by the mandibular nerve.

Retromolar Trigone:

This area is the attached mucosa overlying the ascending rami of the mandible from the level of the posterior surface of the last molar tooth to the apex superiorly, adjacent to the tuberosity of the maxilla. The arterial and nerve supply is similar to that of the lower alveolar ridge.

Floor of the Mouth:

The floor is the semilunar space of the mylohyoid and hypoglossal muscles, extending from the inner surface of the lower alveolar ridge to the undersurface of the lower alveolar ridge to the undersurface of the tongue. It is supplied by the lingual artery through the dorsal lingual branches and sublingual artery and the inferior alveolar artery through its lingual branches. The nerve supply is by the mandibular nerve through the buccal nerve.

Hard Palate:

This is a semi lunar area between the upper alveolar ridge and the mucous membranes covering the palatine process of the maxillary bones. It is supplied by greater palatine artery. The nerve supply is by the greater palatine and nasopalatine branches of the pterygopalatine ganglion (maxillary nerve).

Anterior 2/3rd of the Tongue:

The anterior 2/3rd of the tongue is freely mobile and extends anteriorly from the line of circumvallate papillae to the undersurface of the tongue at the junction of the floor of the mouth. It consists of intrinsic and extrinsic muscles supplied by sublingual artery and inferior alveolar artery through lingual branches. The nerve supply is by the mandibular nerve through the lingual nerve, while taste sensation is supplied by the chorda tympani nerve. All the muscles are supplied by the hypoglossal nerve except for palatoglossus which is supplied by the pharyngeal plexus.

Lymphatic Drainage of the Oral Cavity:

The central part of the lower lip drains into the submental nodes while the rest of the lips drain into the submandibular lymph nodes on

either side. The buccal mucosa drains mainly into the submandibular and preauricular lymph nodes as well as into buccal and mandibular lymph nodes. The anterior part of the lower alveolar ridge has a similar lymphatic drainage. The upper alveolar ridge drains into the submandibular lymph nodes. The hard palate drains into the upper deep cervical and retropharyngeal nodes. The tip of the oral tongue drains bilaterally into submental lymph nodes. Whereas the right and left halves drain mainly to their respective submandibular lymph nodes. A few central lymphatics drain bilaterally.

ANATOMY OF OROPHARYNX

Oropharynx extends from the junction of the hard and soft palate in the superior aspect, the palatoglossal folds in the lateral aspect to the level of the floor of the valleculae.

Base of Tongue:

Extends from the circumvallate papillae to the vallecula covered by non keratinized squamous cell epithelium, lymphoid tissue present below the mucosa forms a part of the Waldeyer's ring. It is continuous with the tonsillar fossa through the tonsillolingual sulcus.

Tonsillar Pillars and Tonsils:

The tonsillar pillars are formed by the palatoglossus fold anteriorly and the palatopharyngeus fold posteriorly, covered by non-keratinising stratified squamous epithelium. Tonsils are oval masses of specialized sub-epithelial lymphoid tissue situated in the triangular tonsillar fossa between the diverging palatopharyngeal and palatoglossal folds. The medial wall has crypts, the surface of which is lined by non – keratinized stratified squamous epithelium.

Soft Palate:

It is a mobile, flexible partition between the nasopharyngeal airway and the oropharyngeal food passage. It forms the roof of the oropharynx. It is formed by the palatine aponeurosis which joins in median raphae. Other muscles which constitute the soft palate include levator palati, palatoglossus, palatopharyngeus and the uvular muscle.

Blood Supply of Oropharynx:

Arterial Supply:

1. Ascending pharyngeal artery from the external carotid artery
2. Ascending palatine and tonsillar branches of the facial artery
3. Greater palatine and pterygoid branches of the maxillary artery

Venous Drainage:

1 Internal submucous plexus of veins

2 External pharyngeal plexus

3 Communicating branches between the external pharyngeal plexus and veins of the dorsum of the tongue, superior laryngeal veins and oesophageal veins.

The pharyngeal plexus drains into the internal jugular vein and anterior facial veins. It also communicates with the pterygoid plexus.

Lymphatic Drainage:

All the lymphatics drain into the upper deep cervical lymph nodes.

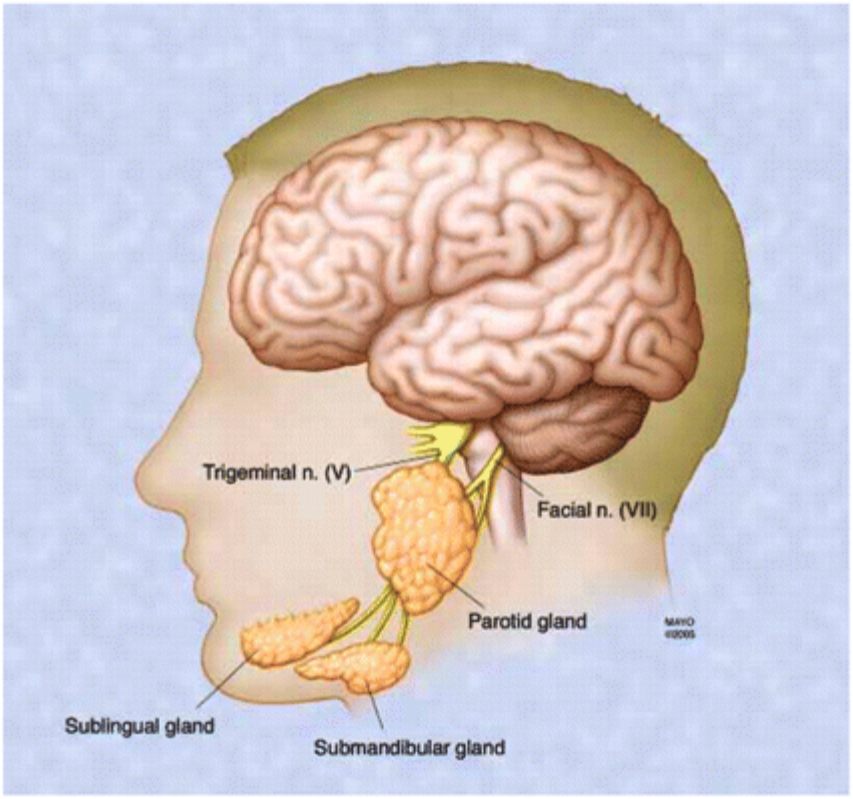
Nerve Supply

All the motor, sensory and autonomic fibres supplying the oropharynx are derived from the pharyngeal plexus situated in the buccophayngeal fascia. In addition secretomotor fibres are supplied from the pterygopalatine ganglion.

Anatomy of salivary glands

Distribution

There are three pairs of major salivary glands, the parotid, submandibular and sublingual, each situated outside the oral cavity but connected to it by a duct or system of ducts. In addition to these, there are collections of salivary tissue just below or within the oral mucosa itself. This tissue is collectively termed the minor salivary glands.



The parotid glands

Gross morphology

Each parotid gland is a slender, lobulated, lozenge shaped structure which has been likened to an inverted pyramid. It has a small superior surface and much larger anteromedial, posteromedial and superficial faces. It anterior border overlies and is densely adherent to the posterior part of the masseter muscle. Superiorly this border is limited by the zygomatic arch, whereas the inferior extent is more variable. Its posterior border abuts onto the tragal cartilage superiorly and extends inferiorly to

overlap the upper part of the sternomastoid muscle. The superior surface is related to the external auditory meatus and the posterior aspect of the temporomandibular joint, while its anteromedial surface is attached to the masseter muscle, posterior border of the mandibular ramus and the medial pterygoid muscle. The posteromedial surface is grooved by the posterior belly of the digastric mucsle, styloid process and its attached muscles and ligaments.

The Capsule

The superficial surface of the parotid gland is covered by skin and fascia. Inferiorly it is also covered by the posterior border of the platysma muscle. The fascia condenses immediately over the gland to form its capsule, which for the most part is thick, tough and inelastic, although inferiorly it becomes thinner. Within the capsule are the superficial parotid lymph nodes and the greater auricular nerve.

The parotid duct

The tree like tributary ducts join near the anterior border of the gland and leave it to pass forward over the superficial surface of the masseter along an imaginary line drawn from the angle of the mouth to the attachment of

the earlobe. The Stenson’s duct opens into the mouth at the parotid papilla opposite the upper second molar tooth.

Submandibular glands

It consists of a large superficial and a smaller deep lobe which are continuous around the posterior border of the mylohyoid muscle. It fills the submandibular triangle in the neck. The medial aspect of the superficial part lies on the inferior surface of the mylohyoid muscle; the lateral surface is covered by the body of the mandible, while its inferior surface lies on both bellies of the digastric muscle. Its inferior surface is covered by the platysma muscle, deep fascia and skin. The deep part of the gland lies on the hyoglossus muscle where it is related superiorly to the lingual nerve and inferiorly to the hypoglossal nerve and deep lingual vein. The capsule of the gland is well defined.

The submandibular duct

Wharton’s duct is formed by the union of several tributaries and is about 5cm in length. It emerges from the deep surface to run in the space between the hyoglossus and mylohyoid muscles to open into a papilla to the side of the lingual frenulum.

The sublingual glands

They lie in the anterior part of the floor of the mouth, between the mucous membrane, the mylohyoid muscle and the body of the mandible close to the symphysis, where each may produce a small depression-the sublingual fossa. The gland has numerous excretory ducts which either open directly onto the mucous membrane or into the terminal part of the submandibular duct.

Special features of individual glands

Parotid: In man as in most mammals the secretory acini of the parotid gland consist entirely of serous cells. The parotid secretion is accordingly a watery serous secretion low in mucin and high in enzymes. The intercalated ducts are long and there are numerous striated ducts.

Submandibular: This is a mixed gland with predominantly serous secretory units. Some of the mucous acini have serous cells at their terminal ends, some have serous demilunes. The ratio of serous to mucous cells is 4:1. The intercalated ducts are short and narrow. The intralobular ducts are branched, longer and more numerous than in the parotid duct system and are lined by two types of cell.

Sublingual gland: This is a complex of small glands although described as a single gland. The acini are mainly pure mucous though some have large serous crescents. There are very few pure serous acini. The ratio of serous to mucous cellular elements is 1:4. The intercalated ducts are very short and some acini open directly into striated intralobular ducts. These intralobular ducts have only one type of ‘striated’ cell. The glands open by some extalobular ducts into the floor of the mouth.

Accessory salivary glands

Many small glands are present in the mucosa and submucosa of the oral cavity. These include.

1. In the oral vestibule: labial glands in the upper and lower lips and buccal glands in the cheeks. These have mixed serous and mucous cells.
2. In the floor of the mouth cavity: small sublingual and glossopalatine glands.
3. On the tongue: anterior lingual and posterior lingual glands

Composition of saliva

Saliva is a colourless opalescent viscous liquid. It consists of water, inorganic constituents of the extracellular fluid, and organic compounds, notably the protein enzymes and mucin.

Its composition shows a great variability in the concentration of the chief individual constituents. The ‘mixed’ saliva collected from the mouth is a mixture of the secretions of main and accessory glands and thus is not very constant in composition. Also it must be realized that the addition of bacterial products of fermentation and putrefaction, food debris, desquamated epithelial cells, and evolution of carbon dioxide into the atmosphere will further modify the composition of the saliva in the mouth.

The main cations of saliva are sodium, potassium, calcium and magnesium and the principal anions are chloride, bicarbonate with smaller concentrations of phosphate and sulphate and traces of thiocyanate, iodide, fluoride and nitrite. The levels of these ions may be influenced by gland of origin, age, sex, rate of secretion, nature of stimulus, plasma concentration, diet and disease.

In general, distinctive characteristics of human saliva are its hypotonicity, and the lower sodium and higher potassium levels relative to the extracellular fluid concentrations.

Organic constituents

The organic constituents of saliva include proteins, polypeptides, amnio acids, urea, uric acid, creatinine, cholesterol, citrate, thiocyanate; vitamins C and B complex. The proteins present are mainly mucin and amylase. In addition many other enzymes, group – specific agglutinogens, albumin, globulin, including blood clotting factors, have been demonstrated in saliva.

Reaction of saliva

The reaction of saliva has been extensively studied both in health and disease. The reaction of saliva is markedly affected by flow rate, by the source of the saliva, and by the method of collection of saliva.

The human unstimulated mixed saliva as secreted is just on the acid side of neutrality. The mean pH is 6.7 with a range of from 5.6 to 7.6. Submandibular resting saliva has a slightly less acid reaction than parotid saliva.

The pH of saliva is extremely sensitive to changes of flow rate. With increased flow in stimulated glands the pH rises and the salivary reaction approximates to the slightly alkaline reaction of plasma.

The cells of the duct system modify the acinar fluid to form the ultimate hypotonic saliva. This ductal activity includes in the main active reabsorption, active secretion and passive transport.

The mechanism of formation of acinar fluid is essentially an active secretory process and not merely a physical process of ultra filtration. The acini are the main source of the water content of all saliva. Saliva contains substances, e.g. amylase and mucins, which are not present in plasma and are therefore synthesized in the gland cells.

The saliva is the end result of the functional activity of several types of cell, particularly a) serous and mucous acinar cells b) striated intralobular duct epithelium c) cells of the main ducts. It is produced by acinar secretion, followed by active ductal reabsorption and secretory activity.

Functions of saliva

Saliva fulfils a number of functions. These include moistening of the mouth, cleansing action, lubrication in swallowing and speech,

buffering, diluent and solvent action, digestive action, thirst mechanism and antibacterial activity.

Innervations of salivary glands

The major salivary glands have a double autonomic supply from the parasympathetic and sympathetic divisions of the autonomic nervous system.

AETIOLOGY OF ORAL AND OROPHARYNGEAL CANCER

Predisposing Factors

Treatment of cancers of the head and neck requires an understanding of their etiology and development. Although the exact cause of these cancers is unknown there is evidence to suggest that they may result from repeated damage to the mucosa by one or more of the factors reviewed below.

1. Smoking tobacco:

The risk of malignancy is 6 times greater for smokers. In addition in South East Asia, beedis, hookah and reverse smoking are also practiced. The relationship between reverse smoking and palatal cancer has been noted. The carcinogens in tobacco smoke are N-nitrosamines, polycyclic hydrocarbons and benzyl–pyrines.

2. Chewing Tobacco (smokeless Tobacco) or Betel nut:

Oral cancer has been observed at the site of placement of the quid, which consists of tobacco, betel leaf, lime, areca nut and sweetening agents. The habit of chewing various forms of tobacco is widely

practiced in India. The probable carcinogens are aracholic acid, slaked lime and tannic acid.

3. Alcohol:

This acts in various ways to cause malignancy. It may act directly as a carcinogen, indirectly by causing vitamin deficiency and alcoholic cirrhosis which prevents detoxification of potential carcinogens. It also acts by altering the metabolism of oral and oesophageal mucosal epithelium and thus facilitates the entry of carcinogens. The risk of malignancy in a smoker who also drinks alcohol is fifteen times higher when compared to a person with neither habit.

4. Syphilis:

The etiological role of syphilis was based on the seropositive reaction seen in patient with oral cancer, as well as the observation that patient with syphilis of the tongue developed malignancy.

5. Diet and Nutrition:

Factors associated with an increased risk of cancer are iron deficiency anaemia (Plummer – Vinson syndrome), Vitamins A and C deficiency, carotenoids. Dietary deficiency can cause epithelial atrophy which renders the epithelium vulnerable to the action of carcinogens.

6. Orodental Factors

Poor oral hygiene, sharp teeth and ill fitting dentures are possible etiological factors and seen mostly over the lateral border of the tongue and the cheek mucosa.

7. Solar Exposure:

The lip is susceptible to mucosal changes due to the absence of melanin pigment which acts as protection against u-v radiation. The ultra violet radiation is especially damaging to the vermilion border of the lower lip.

8. Viral Causes:

Human papilloma virus is capable of transforming cells to a malignant phenotype. HSV–I (herpes simplex virus) and HIV(human immunodeficiency virus) have also been suggested in the pathogenesis of oral squamous cell carcinomas.

9. Genetic:

Mutations in the tumour suppressor genes have been found to be responsible for malignant transformation of tissues. The most commonly identified mutations is in the tumour suppressor gene p53 located on chromosome 17. It plays an important role in the regulation of cell

proliferation. This gene is a potential tumour marker for the future. The epidermal growth factor receptor gene (EGF-R), an oncogene has been associated with squamous cell carcinoma of the head and neck. Nitrosamines can deregulate p53 and oncogenes leading to neoplastic alteration.

PATHOPHYSIOLOGY

Carcinogenesis:

Various substances organic/inorganic, radiation, viral agents, etc. are found to be responsible for carcinogenesis. Of all these smoking and alcohol are the two most important causes for the development of malignancy.

It is found that processed unadulterated tobacco contains at least 2550 known compounds, of which at least 25 compounds are found to be associated with malignancy in humans.

Various theories have been put forth to explain the development of cancer, of which four important theories are.

1. Electrophilicity of chemicals as a determinant of their carcinogenicity. It is proposed that most carcinogens could be considered as pro-carcinogens which change to carcinogens by chemicals due to their electrophilicity and ultimately result in cancer.
2. Free radicals could be responsible in the formation of carcinomas or act as carcinogens themselves and lead to cancer.

3. Carcinogens which cause alteration of DNA methylation and thus result in cancer.
4. Aberrations of DNA repair, wherein carcinogens affect DNA repair, and thus result in cancer.

Stages:

Biological characteristics of the stages in Carcinogenesis

a) Initiation: Irreversible

With constant ‘stem cell potential’ by a mutagen or mutation.

Efficacy is sensitive to xenobiotic and other chemical factors.

Spontaneous occurrence of initiated cells can be demonstrated.

Requires cell division for ‘fixation’

Dose response does not exhibit a readily measurable threshold

Relative effect of initiators depends on the quantization of focal lesions following a definite period of promotion.

b) Promotion: Reversible

Promoted cell population existence is dependent on continued administration of the promoting agent.

Efficacy is sensitive to dietary and hormonal factors. Dose response exhibits a readily measurable threshold and the maximal effect is dependent on the dose of the initiating agent.

Relative effectiveness of promoters depends on their ability with constant exposure to cause an expansion of the progeny of the initiated cell population.

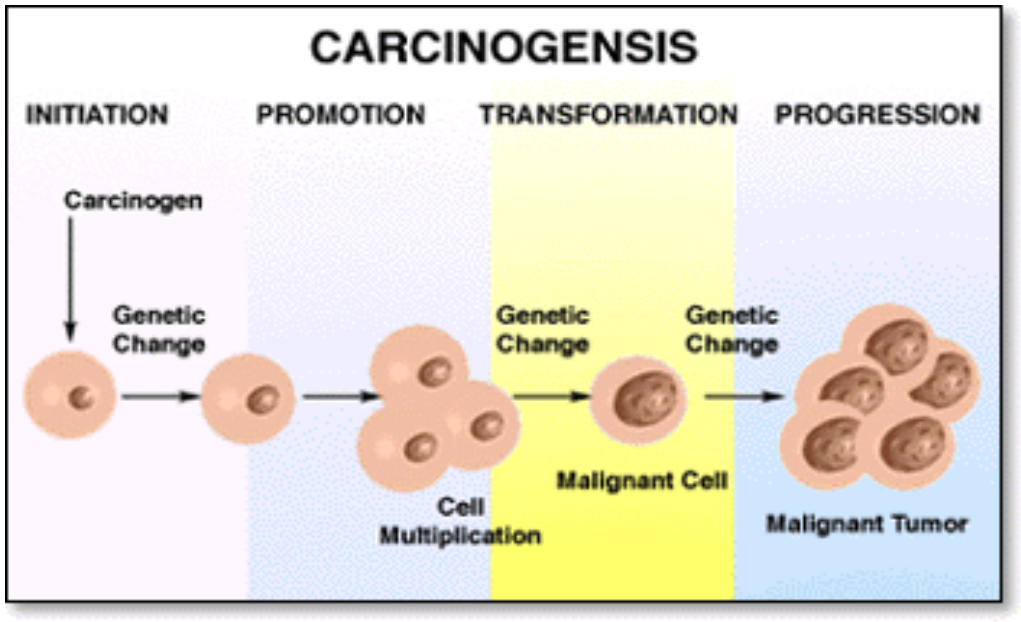
c) Progression: Irreversible

Measurable and/or morphologically discernible alteration in cell genome structure.

Growth of altered cells is sensitive to environmental factors during the early phase.

Benign and/or malignant neoplasms are characteristically seen

"Progressor" agents act to advance promoted cells into this stage but may not be initiating agents.



Spontaneous progression can be demonstrated.

Irrespective of the cause, ultimately malignancy is resulted from subversion of the processes that control the normal growth, location and mortality of cells. This loss of normal control mechanisms arises from the acquisition of mutations in three broad categories of genes.

1. Proto–Oncogenes:

The normal products which are components of signaling pathways that regulate proliferations and which in their mutated form become the dominant oncogene. This may be by mutation or DNA rearrangement or gene amplification due to the action of chemicals, radiation or viruses

2. DNA repair enzymes:

Mutations in DNA repair enzymes promote genetic instability due to faulty or absent regulation of damaged nuclear material.

3. Tumour suppressor genes:

Which generally exhibit recessive behaviour, the loss of function of which leads to deregulated control of the cell cycle progression, cellular adhesion etc. Nearly 50 of such kind are identified of which p53 is most important.

p53: It is a normal transcriptional factor.

(i) It regulates the normal cell growth cycle by activating transcription of genes that control progressions through the cycle and of other genes that cause arrest in 'G1' phase when the genome is damaged and in some cell types promote apoptosis.

Hypoxia occurring in areas of tumours with poor blood supply promotes p53 dependent apoptosis, cells with mutated p53 are resistant to killing by hypoxia.

(ii) It also possesses 3 to 5 DNA exonuclease capacity. This Magnesium dependent activity may be involved in replication

associated DNA repair, p53 acting as a proof reading enzyme for DNA polymerases.

Mutations in the single copy P53 gene are the most frequent genetic changes, shown to be associated with human cancers and point mutations. Deletions or insertions in p53 occur in nearly 70% of all malignant tumours.

Salivary antioxidant system

Saliva is the first biological medium met by external materials taken into the body as part of food, drink, or inhaled volatile ingredients. During evolution, various defense mechanisms developed in the saliva aimed at combating penetrating bacteria, viruses or fungi, and protecting against chemical or mechanical attack. Moreover, even after swallowing, saliva has a mucosal protective capacity within the gastrointestinal tract . An extensive amount of research has been devoted to the immunologic defense mechanism of saliva, primarily based on secretory immunoglobulin A and the protein-enzymatic defense system. That, in turn, is based on the enzyme lysozyme and other components, such as histatin, lactoferrin, proline-rich protein, mucin, etc. The soft tissue integrity defense system, in which the epidermal growth factor plays a

pivotal role, has also been evaluated thoroughly. Recently, the importance of an additional salivary defense system has become clear i.e the antioxidant defence system. Similar to other biological systems, the salivary antioxidant system includes various molecules and enzymes, the most important of which are the uric acid molecule the antioxidant defense system, which appears to lose efficiency with advanced age and the peroxidase enzyme, both of them water-soluble. The lipid-soluble antioxidants carried by lipoproteins, whose concentration in saliva is very low, contribute no more than 10% of the total salivary antioxidant capacity. Uric acid, the most important antioxidant molecule in saliva contributes approximately 70% of the total salivary antioxidant capacity, with the antioxidant role of the ascorbic acid molecule being secondary

Saliva- as a diagnostic sample

Saliva is ultrafiltrate of plasma. In a clinic or lab, saliva is relatively easy to collect in sufficient quantities for analysis and the costs of storage and shipping tend to be lower than those for serum and urine. Saliva is easy to obtain, with less invasion of privacy and ease of adulteration, compared to urine. Salivary sampling protocols are advantageous as they

make for frequent and easy collection of samples by non-invasive NEEDLE-FREE stress free techniques.

Advantages:

Saliva measures free, bioavailable fraction of steroid hormones and drug that have moved out of bloodstream and into the tissue.

- Most reliable measurement of tissue uptake in case of topical hormone supplement.
- Painless, non-invasive, needle free.
- Private, convenient for both patient and doctor.
- Transportation of saliva samples to laboratory requires no special handling.
- Less expensive than conventional blood testing.
- Ease of collection allows for routine monitoring and adjustment of hormone supplement if required.

Clinical presentation and diagnosis

Malignancies arising from the mucosa of the oral cavity are epithelial in origin and are therefore, classified as squamous cell carcinomas more

than 90% of the time. According to the degree of differentiation, three subtypes are defined:

1. well-differentiated squamous cell carcinoma showing more than 75% keratinization
2. Moderately differentiated squamous cell carcinoma with 25-75% keratinization
3. Poorly differentiated squamous cell carcinoma with less than 25% keratinization

The majority of cases are of moderate differentiation. A clear relationship between

histological differentiation and clinical prognosis has not been established, although a lack of differentiation has been associated with more rapid growth and spread. The morphologic classification of squamous cell carcinoma by degree of differentiation is used in the description of the histopathological specimen.

The presentations of oral cancers will be:

- Ulcer
- Growth
- Patches or plaques
- Bleeding
- Halitosis
- Dysphagia or odynophagia
- Pain in mouth or ear
- Neck swellings (lymph nodes)
- Orocutaneous fistulae

The diagnosis of oral cancers is mainly by clinical examination. Tissue biopsy confirmation is done by Edge-wedge biopsy. Other investigations – chest X-ray, CT scan, MRI scan- are done to stage the disease and assess the operability status.

STAGING

The staging of oral cancers is done by TNM staging system, where T denotes Tumor, N stands for Nodal metastases and M for Distal metastases.

TX Primary tumor cannot be assessed

T0 No evidence of primary tumor

Tis Carcinoma in situ

T1 Tumor 2 cm or less in greatest dimension

T2 Tumor more than 2 cm but not more than 4 cm in greatest dimension

T3 Tumor more than 4 cm in greatest dimension.

T4a (Lip)- Tumor invades through cortical bone, inferior alveolar nerve, floor of mouth, or skin of face (ie, chin or nose)

(Oral Cavity)- Tumor invades through cortical bone, into deep extrinsic muscle of tongue (genioglossus, hyoglossus, palatoglossus, and styloglossus), maxillary sinus, or skin of face

T4b Tumor involves masticator space, pterygoid plates, or skull base and/or encases internal carotid artery

NX Regional lymph nodes cannot be assessed

N0 No regional lymph node metastasis

N1 Metastasis in a single ipsilateral lymph node, 3 cm or less in greatest dimension

N2 Metastasis in a single ipsilateral lymph node, more than 3 cm but not more than 6 cm in greatest dimension; in multiple ipsilateral lymph nodes, none more than 6 cm in greatest dimension; in bilateral or contralateral lymph nodes, none more than 6 cm in greatest dimension

N2a Metastasis in single ipsilateral lymph node more than 3 cm but not more than 6 cm in greatest dimension

N2b Metastasis in multiple ipsilateral lymph nodes, none more than 6 cm in greatest dimension

N2c Metastasis in bilateral or contralateral lymph nodes, none more than 6 cm in greatest dimension

N3 Metastasis in a lymph node more than 6 cm in greatest dimension

MX Presence of distant metastasis cannot be assessed

M0 No distant metastasis

M1 Distant metastasis

Treatment

The treatment of oral cancers depends on site, stage of the tumor apart from patient factors. Methods available for the treatment include- Surgery, Radiotherapy, Chemotherapy..

Surgery and radiation therapy are the only curative treatments for carcinoma arising in the head and neck. Chemotherapy is useful in the adjuvant setting; used alone, it is not curative.

The advantages of an operation compared with radiation therapy, assuming similar cure rates, may include the following: (1) a limited amount of tissue is exposed to treatment, (2) treatment time is shorter, (3) the risk of immediate and late radiation sequelae is avoided, and (4) irradiation is reserved for a subsequent head and neck primary tumor, which may not be as suitable for an operation.

The advantages of irradiation may include the following: (1) the risk of a major postoperative complication is avoided, (2) no tissues are removed so that the probability of a functional or cosmetic defect may be reduced, (3) elective irradiation of the lymph nodes can be included with little added morbidity, whereas the surgeon must either observe the neck or proceed with an elective neck dissection (sometimes bilateral depending on the primary site), and (4) the surgical salvage of irradiation failure is probably more likely than the salvage of a surgical failure.

Wide local excision, composite resection with neck dissection are the common surgical procedures followed

Rescue of a surgical failure may be attempted by operation, radiation therapy, or both. Surgical recurrences usually develop at the margins of the resection, in or near the suture line. It is difficult to distinguish the normal surgical scarring from recurrent disease, and diagnosis of recurrence is often delayed. Tumor response to radiation therapy under these circumstances is poor. An operation, radiation therapy, or both, however, may salvage small mucosal recurrences and some neck recurrences.

Indications for postoperative radiation therapy include close (less than 5-mm) or positive margins, extracapsular extension, multiple positive nodes, invasion of the soft tissues of the neck, endothelial-lined space invasion, perineural invasion, and more than 5 mm of subglottic invasion. Chemotherapy may be administered to palliate symptoms in patients with incurable head and neck cancer or as an adjuvant to radiation therapy or surgery, or both, to improve the probability of cure. Adjuvant chemotherapy may be administered before definitive treatment (induction), simultaneously with radiation therapy (concomitant), or after radiation therapy or surgery (maintenance).

AIMS

- To identify a novel Bio-marker in oral cancers using saliva
- To study the relationship between smoking and oral cancers
- To study the different modes of presentation of oral cancers

MATERIALS AND METHODS

Sample collection:

Parafilm (2”X2”)

Gloves and Mask

Short stem plastic funnels (Disposable)

Storage vials

Ice packs and

Labels and marker pens

Sample Processing:

Bradford’s Protein Quantification

SDS : PAGE

Coomassie staining of SDS PAGE

Silver staining

Colloidal coomassie brilliant blue staining

All the patient samples included in the screening analysis study were collected from Government Rajaji Hospital, Madurai, India. A total of 155 samples were collected which includes 82control (Healthy individuals), 39 from patients with oral cancer due to smoking and 34 due to non-smoking.

Stimulated whole saliva was collected with modified protocol of Navazash, 1993, which includes the following steps:

1. Individuals were abstained from eating, drinking, smoking or brushing their teeth for at least 60 min prior to collection.
2. To swallow or discard any accumulated saliva
3. Place a standard piece of parafilm in the mouth and chewed on at a regular pace
4. To expectorate saliva periodically into a collection storage vial (10ml). The volume and physical characteristics of the specimens were recorded. Approximately 5ml of sample were obtained from each individual (Ohshiro *et al*., 2007).
5. Dispose the funnels and parafilm after use. Cap the collection vials and let stand approximately one hour or until and saliva and foam have settled, which may require shaking. Temporary I.D. were created on tubes with permanent I.D. labels, one the sample collection is finished. Place samples in -20 ^{0}C freezer for storage or in the refrigerator.
6. The samples were transported to research area using ice pack coolants.

Sample Processing

Vials were thawed and centrifuged at 3000 rpm for 5-10 minutes and supernatant was collected and cocktail protease inhibitor was added for protein degradation minimization. The supernatant fractions were stored in 250µl aliquots in -20 ^{0}C until analysis. (Modified protocol of Balwant, 2007).

Bradford's protein quantification

Bradford's Reagent

Dissolve 100mg of G-250 in 100ml of absolute/ distilled ethanol or absolute methanol in on a shaker for 60 min. Add 100ml 88% orthophosphoric acid, mix well and make the volume to 500ml with distilled water. Filter through Whatman No. 1. Dilute 1:1 with water and check the A550 against a water blank. The A550 should be approximately 1.1. If not adjust the dilution.

Procedure

Colorimetric Estimation

1. Pipette out increasing amounts(10-100µl) of BSA stock solution (0.2 mg/ml) into clean dry test tubes.
2. Make up the volume to 0.5ml with distilled water. Keep a blank.
3. Simultaneously the saliva samples were added (10-100µl) and made the volume to 0.5ml with distilled water.
4. Add 4.5ml of the dye reagent and mix gently.
5. Read the absorbency at 595 nm after 5 min at room temperature.
6. The concentration of the protein was quantified by plotting optical density value against the BSA standard.

SDS-PAGE

Separating gel (10%) for 10ml

30 % Acrylamide:Bis (29.2:0.8) - 3.3ml

Tris-Hcl pH 8.8 (1.5M) -2.5ml

SDS(10%)- 0.1ml

De-ionised water 4.0 ml

Ammonium persulphate (10%) – 0.1ml

Finally TEMED 0.004 ml

Procedure

Proteins were resolved in SDS-Polyacrylamide gel as described by Ausubel et al., 1989. routinely, 1.5 mm thick gels were cast using a separating gel (10%) and a stacking gel (5%). The separating gel solution was first poured into a sealed glass plate cassette. Water saturated n-butanol was layered on top of the separating el and the gel was set to polymerize for 30 min at room temperature. After polymerisation the butanol layer was removed carefully and the exposed end was rinsed with 0.1% SDS. Stacking gel solution was poured onto the separating gel up to the cassette leaving 1cm. comb was inserted into the cassette leaving 1cm distance between the bottom of the slots and top of the separating gel. After the polymerization of the stacking gel, the comb was carefully removed. The slots were cleaned by rinsing with 1X TGS buffer. The samples were heated in a boiling water bath for 3min, loaded into the slots and the slots were filled with 1XTGS buffer and a constant current of 21 mA/ gel was applied.

Coomassie staining of SDS-PAGE

After the completion of electrophoresis, the gel cassette was dismantled and the gel was immersed in coomassie blue staining solution and agitated slowly for 2hour. The gel was then left in destaining solution for 2 to 4 hours with 2 to 3 changes of the solution. After destaining, the gels were stored in 7% acetic acid.

Silver staining

Reagents

1. fixing solution: 40% ethanol +10 acetic acid

2. Rinsing solution: 30% ethanol

3. Sensitizing solution: 0.02% sodium thiosulfate

4. Staining solution: 0.1% silver nitrate

5. Developing solution: 6% sodium carbonate+ 0.04 formalin (add just before the use from stock 40% or 37%)

6. Stopper solution: 5% acetic acid

Procedure

The gel was placed in fixing solution and gently shaked for 1 hr in a rotary shaker. After incubation, the fixing solution were removed without touching the gel and rinsed with 30% alcohol for 20 min with gentle shaking, washed thrice in water at each 10 min and sensitizing solution was added and left it from 1min. Again the gel was washed thrice in water at each 30 seconds intervals. The gel was stained by adding pre-cooled staining solution with gentle shaking in cold room for 30 min, washed with water(10 seconds) and developed in a developing solution(fresh developer and gentle shaking until the protein/ peptide spots appeared clearly. The gel was washed with water thrice for 10min and stored in 1% acetic acid.

Colloidal coomassie brilliant blue staining

Reagents:

Solution I : The solution I consisted of 16 ml of orthophosphoric acid, 868 ml of distilled water and 800 gm of ammonium sulphate and they were mixed well

Solution II : The solution II was prepared with 5% CBB G250in distilled water and 16ml of solution I just prior to use.

Solution III: The solution III was prepared freshly by slowly adding 200ml of methanol to the solution II to give a final concentration of 0.08% CBB G250, 1.6 % orthophosphoric acid, 8% ammonium sulphate and 20% methanol. All solutions were prepared on a rotary shaker with gentle shaking.

Procedure

Solution III was poured in a plastic tray and the gel was placed and incubated for overnight with gentle shaking in a rotary shaker.

Destaining

Overnight stained gel was destained with several washes with double distilled water until the background get transparent.

RESULTS AND DISCUSSION

Sample collection:

Type of sample	No. of Samples	Age groups(range)	remarks
Control	82	25-70 years	-----
Smoker oral cancer	34	33-67 years	Poor oral hygiene
Non smoker oral cancer	32	50- 65 years	Poor oral hygiene
Oral cancer without any habit	7	45-55 years	Poor oral hygiene

Whole stimulated saliva were collected from the individuals of 4 different category, to make a comparative study among them. From the Above Table it is found that the individual are more prone to oral cancer are due to poor oral hygiene which is found to be the main reason as the individuals with the habit of smoking or chewing tobacco are also found to have oral cancer. While considering the occurrence of cancer with age, it is found that the age group ranges from 33 to 67 years. While, the age group ranging from the 55 to 65 are more susceptible for oral cancer.

The secretion of saliva volume differs for individual to individual, so we used acid candies for collection of saliva samples.

SDS-

A comparative study was made to check the protein profile using Sodium dodecyl sulfate - polyacrylamide gel electrophoresis. The stored stimulated whole saliva was quantified and denatured. 250 µg of protein sample was loaded to SDS-PAGE gel for separation and the gel was stained with coomassie stain for visualization of protein bands, and documented in Alpha Imager 1200.

The protocol was standardized as the proteins in the samples were degraded and were unable to perform the protein studies. So, we started using cocktail protease inhibitor to avoid the problem of degradation (Fig1).

Fig 1

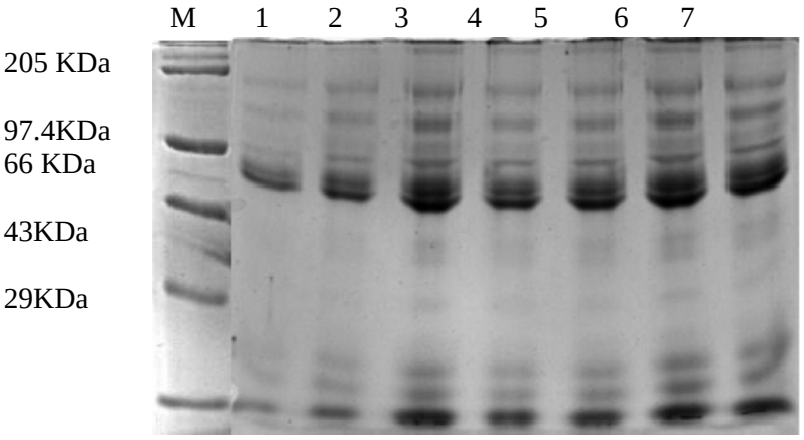


Fig.1 Fresh and stored saliva samples from different Normal (control) individuals were subjected to 12%SDS-PAGE and visualized via Coomassie blue staining. lanes A- marker, M-Male, F-Female, S-Stored sample(80µl), Fresh sample F&M- 50µl

As the samples collected included the male and female, so SDS-PAGE was performed to compare the protein finger printing of the male and female sample and found to have similar pattern of protein banding(Fig 2).

Fig. 2

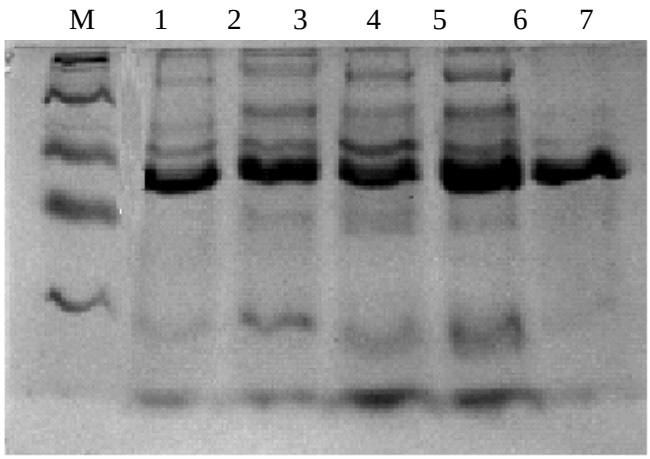


Fig.2 Test saliva sample were subjected to 12%SDS-PAGE and visualized via Coomassie blue staining. Lanes M- Molecular weight marker, Lanes 1-5 oral cancer-smoker saliva sample, C- Healthy control.

Oral cancer whole saliva samples were screened to study the protein profile of oral cancer smokers sample in comparison to control samples. From the study, we found that the expression of protein level were found to be different in each sample Fig. 3 & Fig. 4 when compared to the control samples. Some of the proteins in the samples are found to be over expressed, which reveals that a particular protein cannot be chosen based on the SDS-PAGE for identification of Oral Cancer Biomarker.

Fig 3

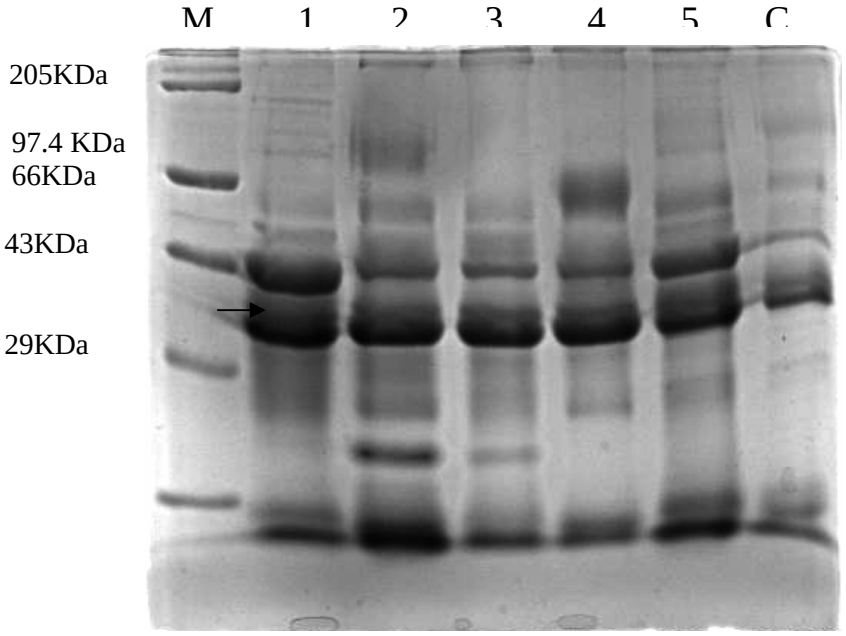


Fig 4

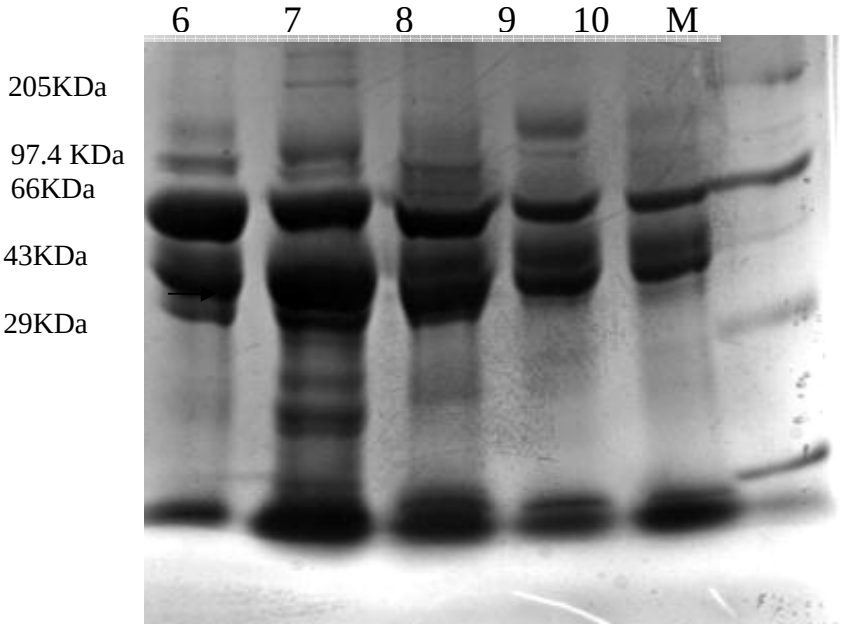


Fig.3 & 4. Oral cancer saliva sample were subjected to 12%SDS-PAGE and visualized via coomassie blue staining Lanes M-Molecular weight marker, Lanes 6-10 oral cancer-smoker sample, C-Healthy control

Simultaneously the SDS-PAGE analysis was performed in 12% gel to check whether the pattern of the protein banding is similar in oral cancer smokers sample to that of non-smokers oral cancer sample. As a result we found the protein expression of the samples of oral cancer non-smokers and the oral cancer without any habit were found to be under expressed when loaded the same amount of quantified whole saliva protein. (Fig.5)

Fig 5

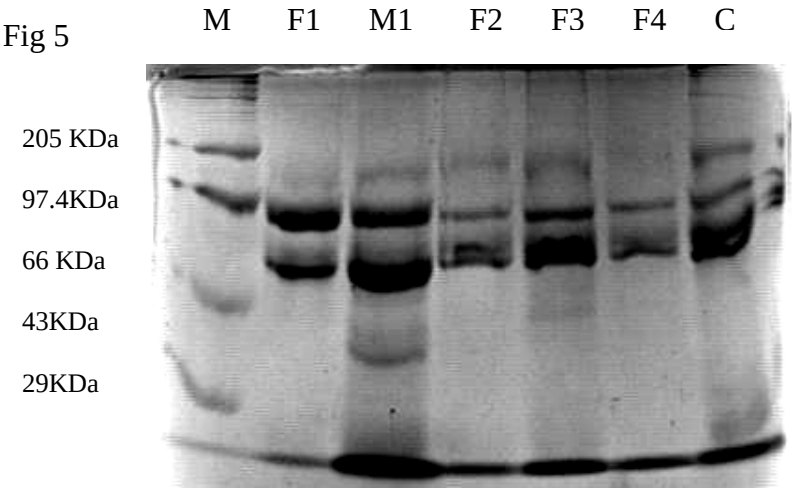


Fig.5 Non-smoker test saliva sample were subjected to 12%SDS-PAGE and visualized via coomassie blue staining. Lanes M-Molecular weight marker, Lanes 6-10 oral cancer-nonsmoker sample, C-Healthy control

Further, Two Dimensional Electrophoresis (2DE) was performed as a attempt to know the complete proteome profile. We performed 2DE which include the first dimension of Isoelectric Focusing at a pH range of 3-10, 11CM pH strip which was followed by the second dimension of 12.5% separating PAGE gel and the silver stained or colloidal coomassie stained to visualize the protein spots. 250 µg of quantified protein sample using Bradford's method and found the proteins were visible only the pH range of 4-8.

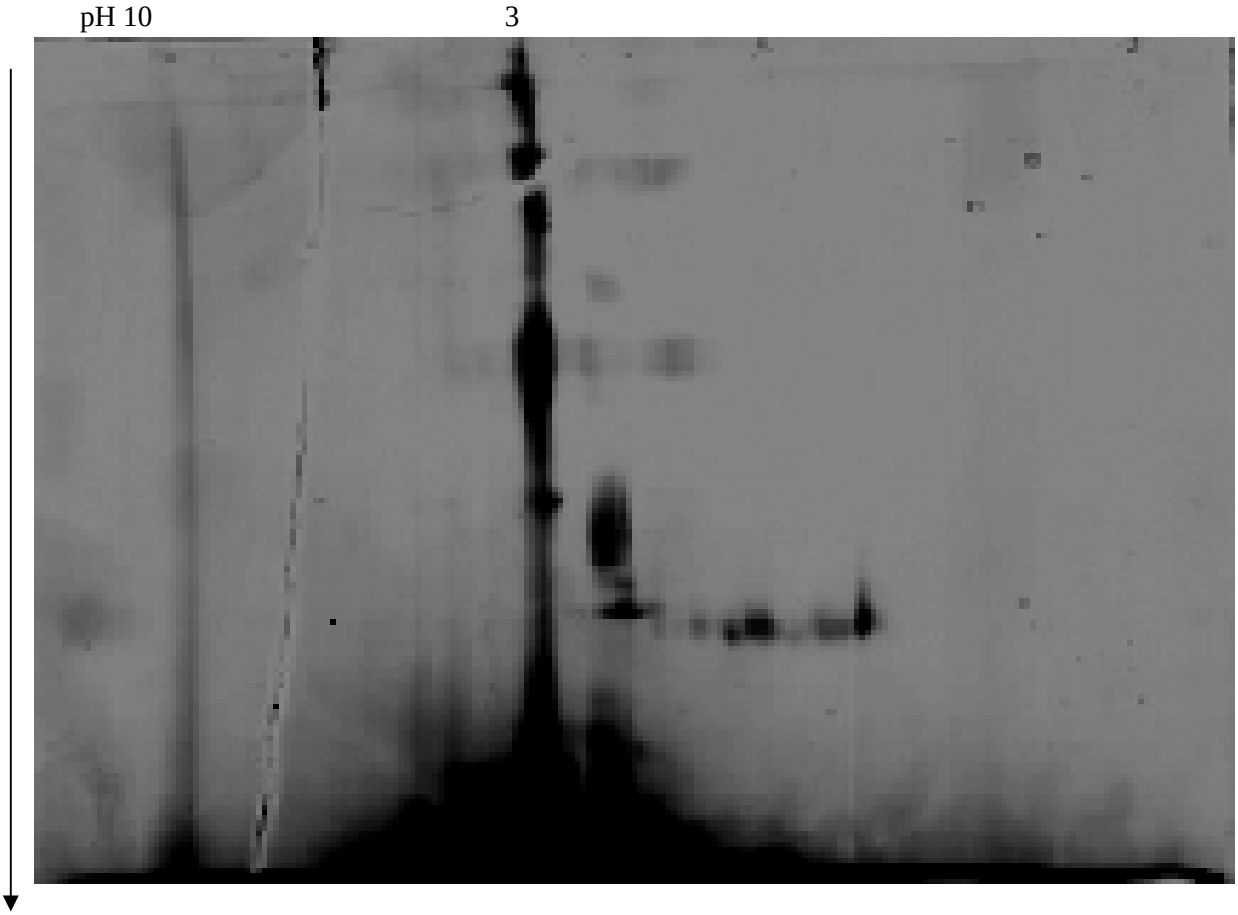


Fig.6 Two-dimensional gel electrophoresis of salivary glandular secretions by isoelectric focusing with pH range 3-10 in the horizontal dimension. Proteins from control(100µg). gel was silver stained.

CONCLUSIONS

1. The protein samples are highly degradable so the analysis should be done immediately after sample collection or cocktail protease inhibitor should be added to avoid degradation.
2. Protein banding pattern of male and female control samples were similar in 12 % SDS-PAGE analysis.
3. Over-expression level of proteins was found in smokers oral cancer patients when compared to control sample and also additional bands was found in three oral cancer sample, which shows that the expression level differs from individual to individual.
4. The under expression of protein in female non-smokers oral cancer patient sample was found when compared to the control female and male smokers patient sample.
5. 2DE protocol for whole saliva sample is performed which lies in 4-8 pH range when the screened at 3-10 pH range.

FUTURE PLANS

- Comparative proteome analysis of control and oral cancer saliva sample, using 2Dimensional Electrophoresis and MALDI-TOF/MS analysis at a pH range around 4 -8 pH has to be screened.

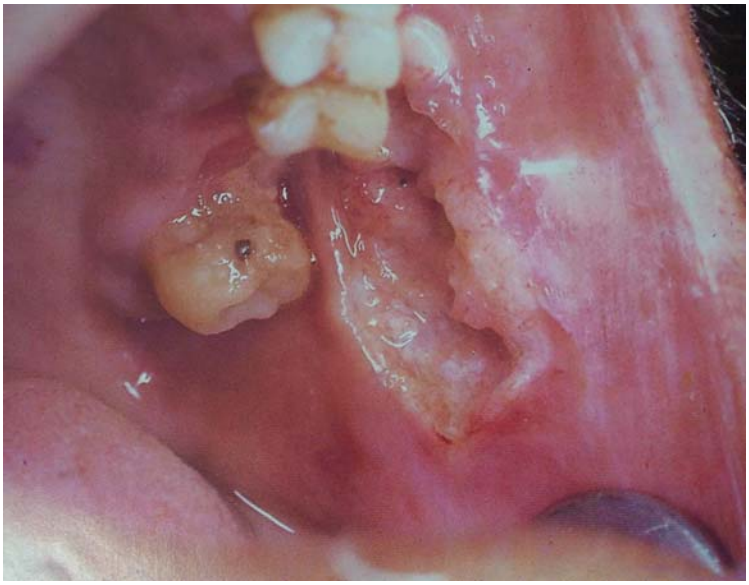
When the target protein biomarker is identified, it can be used for diagnosis, prognostic or therapeutic purposes.

65Yr MALE PATIENT WITH ULCEROPROLIFERATIVE GROWTH AT ® ANGLE OF MOUTH

57Yr MALE PATIENT WITH ADVANCED CARCINOMA ® CHEEK WITH SKIN INVOLVEMENT (OROCUTANEOUS FISTULA)

A 49Yr FEMALE PATIENT WITH ADVANCED CARCINOMA ® CHEEK WITH MANDIBULAR AND SKIN INVOLVEMENT

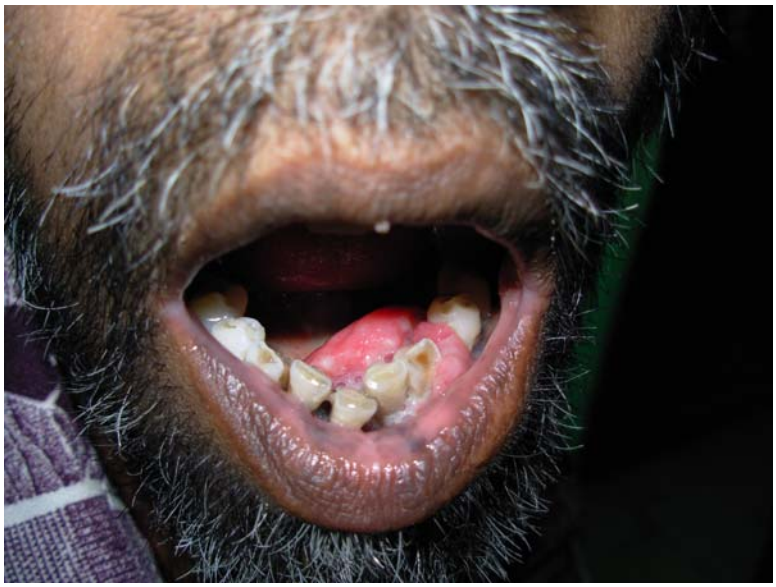
A 51Yr MALE PATIENT MALIGNANT ULCERATIVE LESION IN ® CHEEK



A 62Yr MALE PATIENT WITH ULCEROPROLIFERATIVE LESION OVER (L) LATERAL BORDER OF THE TONGUE

A 58Yr MALE PATIENT WITH CARCINOMA LOWER LIP WITH BILATERAL LOWER LIP AND ALSO BILATERAL UPPER LIP INVOLVEMENT

A 64Yr MALE PATIENT WITH ULCEROPROLIFERATIVE GROWTH IN FLOOR OF MOUTH ON (L) SIDE



PROFORMA

IDENTIFICATION OF NOVEL BIOMARKER FOR ORAL CANCER USING SALIVA.

Patient details

Sample no.:

Date of admission:

Name :

Age/sex :

I.P no/unit :

Address :

Complaints:

Significant history/ comorbid conditions:

Smoking:

On exmn:

Gross

Microscopy

Diagnosis:

S.No	Name	Age	Sex	IP No.	Date of Admission	Father/Husband	Address	Ward	Complaints							Smoking				Others		Comorbidity	Diagnosis	
									Ulcer	Growth	Bleeding	Pain	Dysphagia	Trismus	Period (months)		Type	Period(years)	Quantity/Day	Alcoholic	Beetel nut			
1	VAYANA PERUMAL	65	MALE	100462	6/3/2008	ATHISAMY	MADURAI	SUR4		✓	✓				4	✓	BEEDIES	25	4 TO 6				CARCINOMA LEFT LOWER GINGIVOBUCCAL SULCUS	T3N1M0
2	SUBRAMANI	60	MALE	30130	13/3/2008	PALANI	MADURAI	ENT2		✓		✓	✓		2	✓	BEEDIES	25	20 TO 25				CARCINOMA LEFT LATERAL BORDER OF TONGUE	T4N1M0
3	NALLA KAMAN	58	MALE	32998	13/3/2008	VEERATHEVAR	MADURAI	ENT1				✓	✓		1	✓	BEEDIES	20	12TO15				CARCINOMA POSTERIOR 1/3RD OF TONGUE	T2N1M0
4	VASANTHA	57	FEMALE	54784	22/6/2008	MANI	MADURAI	ENT2					✓		6							DIABETES	CARCINOMA LEFT TONSIL	T4N2bM0
5	MURUGAN	33	MALE	11153	3/4/2008	SUBBIAH CHETTIAR	TIRUNELVELI	SUR4		✓					3	✓	BEEDIES	25	12TO15				CARCINOMARIGHT CHEEK	T3N1M0
6	SUBRAMANI	68	MALE	39202	14/4/2008	NAGAPPA	ARUPPUKOTAI	S.ONCO	✓	✓					1	✓	BEEDIES	10	5TO6	✓	✓	DIABETES	CARCINOMA TONGUE RIGHT SIDE(OPERATED-POSITVE MARGINS)	
7	SOLAIAPPAN	62	MALE	37502	15/4/2008	KALIAPPAN	MADURAI	ENT1		✓				✓	8	✓	BEEDIES	10	10TO12				CARCINOMA RIGHT TONSIL	T2N1M0
8	NAINA MOHAMMED	67	MALE	39664	15/4/2008	MD MEERAN	SIVAGANGAI	ENT2				✓	✓		6	✓	BEEDIES	25	25TO30	✓			CACINOMA OROPHARYNX RIGHT SIDE	T4N1M0
9	SEKAR	55	MALE	52169	20/6/2008	PERUMALSAMY	SIVAKASI	SUR4				✓			3	✓	BEEDIES	20	25TO30	✓		HYPERTENSION	CARCINOMA RIGHT VALLECULA	T4N2bM0
10	CHINNAVELUSAMY	65	MALE	53685	22/6/2008	PERIYASAMY	MADURAI	ENT2				✓			4	✓	BEEDIES	30	30TO32				CARCINOMA LEFT TONSIL	T3N2M0
11	THIRUMALASAMY	73	MALE	59072	14/7/2008	GOPALSAMY	THENI	S.ONCO	✓						6	✓	CIGARETTES	10	3TO5				CARCINOMA LEFT HARD PALATE WITH MELANOPLAKIA	T2N0M0
12	PALANISAMY	62	MALE	59900	5/7/2008	KARUPPIAH	MADURAI	S.ONCO	✓						6	✓	BEEDIES	40	15TO20			?TVGJ done	CARCINOMA LEFT CHEEK BUCCAL MUCOSA	T3N0M0
13	ALLIS	56	FEMALE	28764	11/3/2008	SABISTON	MOONAR	SUR6	✓			✓			12						✓		CARCINOMA LEFT CHEEK BUCCAL MUCOSA	T3N1M0
14	MARIMUTHU	63	FEMALE	29065	19/3/2008	VELLPPAMUPANAR	THENI	SUR1		✓		✓			12						✓		CARCINOMA RIGHT CHEEK BUCCAL MUCOSA	T4aN2cM0
15	CHITTAL	54	FEMALE	37273	31/3/2008	ADAIKAN	PUDUKOTAI	SUR7				✓	✓	✓	60						✓		CARCINOMA LEFT LOWER ALVEOLUS	T4bN2cM0
16	PAPPA	65	FEMALE	34370	31/3/2008	AIYYAR	NILAIKOTAI	SUR5		✓		✓			6						✓		CARCINOMA LEFT CHEEK BUCCAL MUCOSA	T4N1M0
17	MARIAPPAN	60	MALE	35128	12/4/2008	PALANIJOTHI	THENI	S.ONCO	✓						2	✓	BEEDIES	30	20TO25	✓			CARCINOMA LEFT BONY ALVEOLUS	T4aN1M0
18	PITCHAIMMAL	50	FEMALE	39658	12/4/2008	MANI	MADURAI	S.ONCO						✓	3								CARCINOMA TONGUE LEFT SIDE	T2/3N2M0
19	SONAI MUTHU	55	MALE	33384	13/3/2008	SIVALINGAM	SIVAKASI					✓	✓		4	✓	BEEDIES	20	15TO18	✓			CARCINOMA LEFT OROPHARYNX	T2N1M0
20	MUTHIAH	47	MALE	60013	14/7/2008	NALLAN	DINDIGUL	S.ONCO				✓			6						✓		CARCINOMA RIGHT SIDE TONGUE	T2N2bM0
21	VELUTHAI	60	FEMALE	59816	14/7/2008	VELLAIYAN	DINDIGUL	S.ONCO		✓		✓			10						✓		CARCINOMA LEFT BUCCAL MUCOSA	T1N0M0
22	PONNUSAMY	50	MALE	39834	15/4/2008	SAMIKANNUSERVAI	MADURAI	ENT1		✓					4	✓	BEEDIES	30	15TO20	✓		TUBERCULOSIS	CARCINOMA RIGHT POSTERIOR 1/3RD TONGUE	T2N1M0
23	CHANDIRAMANI	55	MALE	47005	22/6/2008	JACOB	NAGERCOIL	ENT2		✓					6	✓	BEEDIES	40	7PACKS	✓			CARCINOMA LEFT POSTERIOR 1/3RD TONGUE	T4N2bM0
24	PALANIYAMMAL	50	FEMALE	79965	28/9/2008	NAGAMUTHU	DINDIGUL	S.ONCO	✓	✓					4						✓	MITRAL STENOSIS	CARCINOMA LEFT LOWER ALVEOLUS	T4N2MO
25	RAMAYEE	50	FEMALE	80631	29/8/2008	RAMASAMY	DINDIGUL	S.ONCO	✓	✓			✓	✓	3						✓		CARCINOMA FLOOR OF MOUTH	T4N1M0
26	ELIZABETH	65	FEMALE	80019	28/9/2008	MICHAEL	VIRUDHANAGAR	S.ONCO	✓						4						✓		CARCINOMA LEFT BUCCAL MUCOSA, RETROMOLAR TRIGONE	T4N1M0
27	MARIAPPAN	65	MALE	35128	18/3/2008	PALANIJOTHI	THENI	S.ONCO		✓		✓			6	✓	BEEDIES	20	5PACKS	✓			CARCINOMA LEFT UPPER ALVEOLUS	T4N2M0
28	KATHAYEE	50	FEMALE	38216	3/4/2008	ALAGAR	MADURAI	SUR7	✓		✓				5								CARCINOMA BUCCAL MUCOSA LEFT SIDE	T2N1M0
29	SUBRAMANI	68	MALE	39202	8/4/2008	RAIKAPPAN	VIRUDHANAGAR	S.ONCO	✓	✓					3						✓		CARCINOMA TONGUE LEFT SIDE	T2N0M0
30	PITCHAIMANI	50	FEMALE	39658	10/4/2008	MANI	MADURAI	S.ONCO		✓					8	✓	CIGARETTES	20	5TO8				CARCINOMA TONGUE RIGHT SIDE	T2N1M0
31	LAKSHMANAN	67	MALE	39689	10/4/2008	RAJAGOPAL	MADURAI	S.ONCO		✓					6	✓	BEEDIES	23	15TO20	✓	✓	DIABETES	CARCINOMA TONGUE LEFT SIDE	T3N2bM0
32	CHINNAIAH	65	MALE	40737	15/4/2008	MANKABAKAN	DINDIGUL	S.ONCO		✓					6	✓	BEEDIES	15	15TO20	✓			CARCINOMA LEFT UPPER ALVEOLUS	T4N2cM0
33	KARUPPANAN	40	MALE	41179	17/4/2008	VELLAIKANNU	SIVAGANGAI	ENT2		✓					6	✓	CIGARETTES	15	10TO12		✓		CARCINOMA PAROTID RIGHT SIDE	T2N1M0
34	ABDUL KHADER	65	MALE	43633	29/4/2008	SULAIMAN	DINDIGUL	ENT2		✓		✓			6	✓	BEEDIES	25	18TO20				CARCINOMA RIGHT UPPER ALVEOLUS	T3N2bM0
35	VEERAMMAL	50	FEMALE	44067	1/5/2008	ALAGAR	DINDIGUL	S.ONCO	✓	✓					4						✓		CARCINOMA BUCCAL MUCOSA LEFT SIDE	T2N1M0
36	INDRAMMAL	70	FEMALE	44093	1/5/2008	PALANICHAMY	THENI	S.ONCO	✓	✓					5								CARCINOMA TONGUE LEFT SIDE	T2N1M0

37	GANESAN	29	MALE	45311	7/5/2008	AIYANAR	MADURAI	SUR7		✓					3	✓	BEEDIES	10	20TO30				CARCINOMA TONGUE LEFT SIDE	T3N2bM0
38	ANNAMALAI	30	MALE	49549	27/5/2008	VELUSAMY	TANJORE	S.ONCO		✓					5	✓	BEEDIES	26	15TO20	✓	✓		CARCINOMA POSTERIOR 1/3RD OF TONGUE	T4N2cM0
39	RAKKIAH	58	MALE	50045	29/5/2008	RAMASAMY	ANDIPATTI	S.ONCO	✓						2								CARCINOMA BUCCAL MUCOSA LEFT SIDE	T3N1M0
40	KOKILA	50	FEMALE	500905	3/6/2008	KUMAR	VIRUDHANAGAR	S.ONCO		✓		✓			6								CARCINOMA HARD PALATE LEFT SIDE	T4bN2cM0
41	RAJKUMAR	55	MALE	51382	11/6/2008	RAMASAMY	ANDIPATTI	S.ONCO		✓		✓	✓	✓	6	✓	BEEDIES	29	20TO22			DIABETES, HYPERTEN	CARCINOMA BUCCAL MUCOSA RIGHT SIDE	T3N2bM0
42	KUMAR	47	MALE	521166	9/6/2008	SEVUGAN	MADURAI	S.ONCO	✓						1	✓	CIGARETTES	14	10TO12		✓	DIABETES	CARCINOMA OROPHARYNX	T4bN2cM0
43	PANDIAMMAAL	63	FEMALE	54539	19/6/2008	ARMUGAM	SELLUR	S.ONCO	✓						18						✓		CARCINOMA LEFT UPPER ALVEOLUS	T4bN2cM0
44	PITCHAI	67	MALE	53293	24/6/2008	CHIDAMBARAM	MADURAI	S.ONCO	✓	✓					7						✓		CARCINOMA BUCCAL MUCOSA LEFT SIDE	T3N1M0
45	PANDIARAJAN	57	MALE	57639	1/7/2008	MAYANDIPILLAI	MADURAI	S.ONCO		✓					5	✓	BEEDIES	15	15TO20				CARCINOMA BUCCAL MUCOSA LEFT SIDE	T3N2bM0
46	LAKSHMI	53	FEMALE	58449	4/7/2008	KARUPPAIAH	MADURAI	S.ONCO	✓	✓	✓	✓			10								CARCINOMA LIP LEFT LOWER SIDE	T2N1M0
47	MUTHIAH	42	MALE	60013	12/7/2008	SAILAR	DINDIGUL	S.ONCO		✓					9						✓		CARCINOMA TONGUE RIGHT SIDE	T2N1M0
48	PALANISAMY	47	MALE	60031	10/7/2008	ALAGARSAMY	DINDIGUL	S.ONCO		✓				✓	3	✓	BEEDIES	20	20TO22		✓	HYPERTENSION	CARCINOMA LEFT UPPER ALVEOLUS	T3N2aM0
49	RAMAKRISHNAN	56	MALE	61316	15/7/2008	GANAPATHY	VIRUDHANAGAR	S.ONCO		✓					24						✓		CARCINOMA PAROTID LEFT SIDE	T3N1M0
50	BALAKRISHNAN	55	MALE	64276	26/7/2008	NELLAIANANTHAN	MADURAI	ENT1	✓						8	✓	BEEDIES	25	3PACKS	✓			CARCINOMA TONGUE LEFT SIDE	T2N0M0
51	MEENATCHI	70	FEMALE	64892	29/7/2008	ZAKIR	SELLUR	ENT1				✓			3						✓		CARCINOMA RIGHT LOWER ALVEOLUS	T2N2aM0
52	SOLAIAPPAN	70	MALE	74797	4/9/2008	SOLAI	MADURAI	SUR5	✓						7	✓	BEEDIES	25	1PACK		✓		CARCINOMA TONGUE RIGHT SIDE	T2N1M0
53	ALAGAMMAL	60	FEMALE	80027	23/9/2008	PARAMAN	MELUR	SUR3	✓	✓					5								CARCINOMA RIGHT UPPER ALVEOLUS	T3N2cM0
54	ARMUGAM	52	MALE	80639	25/9/2008	SANNASI	MADURAI	S.ONCO		✓					1	✓	BEEDIES	23	12TO15			DIABETES	CARCINOMA BUCCAL MUCOSA LEFT SIDE	T2N2bM0
55	LATCHMIAMMAL	65	FEMALE	80652	25/9/2008	ALAGARSAMY	KARIYAPATTI	S.ONCO		✓					5						✓		CARCINOMA LEFT LOWER ALVEOLUS	T2N2bM0
56	PONNUTHAI	60	FEMALE	82124	30/9/2008	SUNDARAM	MADURAI	ENT2		✓				✓	[illegible]						✓		CARCINOMA LEFT UPPER ALVEOLUS	T4aN2cM0
57	BALAMURUGAN	51	MALE	98903	16/12/2008	VAIRAVALAL	DINDIGUL	S.ONCO		✓					18					✓	✓		CARCINOMA PAROTID LEFT SIDE	T3N1M0
58	BOOPATHI	55	MALE	99788	24/12/2008	GANDHICHETTIAR	THENI	S.ONCO	✓	✓					1	✓	BEEDIES	30	4PACK	✓			LEFT SUBMANDIBULAR GLAND TUMOR	T2N1M0
59	AMMAL	48	FEMALE	7534	29/1/2009	RAJI	THENI	S.ONCO	✓	✓		✓	✓		4								CARCINOMA BUCCAL MUCOSA LEFT SIDE	T2N2bM0
60	KAMATCHIAMMAL	75	FEMALE	7568	29/1/2009	PANJARU NAIDU	THENI	S.ONCO	✓						6						✓		CARCINOMA BUCCAL MUCOSA RIGHT SIDE	T2N1M0
61	MADHAVAN	60	MALE	26177	2/3/2009	SHANMUGAM	SIVAGANGAI	S.ONCO	✓						3	✓	CIGARETTES	10	8TO10				CARCINOMA TONGUE LEFT SIDE	T2N1M0
62	MOORTHI	55	MALE	30764	20/3/2009	GANDHI	THENI	S.ONCO		✓					10					✓	✓		LEFT SUBMANDIBULAR GLAND TUMOR	T3N2bM0
63	AANDI	61	MALE	34220	31/3/2009	AYYAR	DINDIGUL	S.ONCO		✓		✓			2	✓	BEEDIES	25	25TO30	✓	✓		CARCINOMA LEFT LOWER ALVEOLUS	T3N2bM0

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